Effect of cold-dryness on pulmonary and immunologic function in chronic obstructive pulmonary disease model rats

Zhen Gao, Fengsen Li, Halmurat Upur, Jiang Min, Wang Jing, Jing Jing, Dan Xu

Zhen Gao, Fengsen Li, Jiang Min, Wang Jing, Jing Jing, Dan Xu, National Clinical Research Base of Traditional Chinese Medicine, Traditional Chinese Medicine Hospital Affiliated to Xinjiang Medical University, Urumqi 830000, China

Halmurat Upur, National Clinical Research Base of Traditional Chinese Medicine, Traditional Chinese Medicine Hospital Affiliated to Xinjiang Medical University, Urumqi 830000, China; Institute of Traditional Uighur Medicine, Xinjiang Medical University, Urumqi 830011, China

Supported by the National Nature Science Fund of Xinjiang (Experimental study of pathogenesis characteristics cold-dryness chronic obstructive pulmonary disease in Xinjiang, No. 2012211835)

Correspondence to: Prof. Halmurat Upur, National Clinical Research Base of Traditional Chinese Medicine, Traditional Chinese Medicine Hospital Affiliated to Xinjiang Medical University, Urumqi 830000, China. Halmurat@263.net
Telephone: +86-13999880996, +86-991-5813580
Accepted: August 29, 2013

Abstract

OBJECTIVE: To study the effects of cold-dryness on pulmonary and immunologic function of peripheral T-lymphocytes in chronic obstructive pulmonary disease (COPD) model rats, and to provide references for the prevention and treatment of cold-dryness COPD in the Xinjiang region.

METHODS: The COPD model was established with an elastase drip into the trachea combined with smoking. The cold-dryness COPD model was developed by stressing with a cold-dry environment. Success of the model was determined by observation of pathologic lung sections. Rats were sacrificed by exsanguination from the femoral artery and changes of peripheral blood CD4+, CD8+, and CD4+/CD8+ were detected by flow cytometry. Data were analyzed with SAS 11.5 statistical software.

RESULTS: On the ninetieth day after ending the experiment, Peak expiratory flow in the cold-dryness COPD group was lower than that in the COPD and normal control groups (P<0.01). The time of inspiration in the cold-dryness COPD group was higher than that in the COPD and normal groups (P<0.05). Time of expiration (Te) in the cold-dryness COPD group was higher than that in the COPD and normal groups (P<0.01). 50% tidal volume expiratory flow (EF50) in the cold-dryness COPD group was lower than that in the COPD and normal groups (P<0.01), and EF50 in the COPD group was lower than that in the normal group (P<0.05). CD4+ content of peripheral blood in the cold-dryness COPD group was lower than that in the COPD and normal groups (P<0.05). CD8+ content in the cold-dryness COPD and COPD groups was higher than that in the normal control group (P<0.01), and CD8+ content in the cold-dryness COPD group was higher than that in the COPD group (P<0.01). CD4+/CD8+ content in the cold-dryness COPD group was lower than that in the COPD group (P<0.01), and CD4+/CD8+ in the cold-dryness COPD group and the COPD group was lower than that in the normal control group (P<0.01), and CD4+/CD8+ in the cold-dryness COPD group was lower than that in the COPD group (P<0.05).

CONCLUSION: In the cold-dryness COPD model, CD8+ increased and CD4+/CD8+ decreased. Moreover, cold-dryness may aggravate this state. The effects of cold-dryness on pulmonary function mainly manifested as prolongation of Te and decrease of EF50, which could be one of causes of cold-dryness environment in the northwest of China leading to COPD with region characteristics.
INTRODUCTION

World Health Organization, in a report on environment burden of disease, estimates that 24% of global disease burden and 23% of all death are caused by environmental hazards. Decreases in environment temperature can weaken preventive capabilities of air passages, leading to increased reactivity of air passages and inducing chronic bronchitis. The combination of cold with dryness will dry out respiratory mucosa and decrease its elasticity, immunity, and ability to remove foreign objects. This leads to growth of bacteria and viruses in the respiratory tract.

Cold air can exacerbate shortness of breath in patients during exercise, leading to decreased exercise capability, decreased quality of life, and increased hospital visits. Xinjiang is located in the northwest of China. Sa Wen, in the book entitled "Yin Yang Ying Xiang Da Lun" said that ‘dryness in the west,’ ‘cold in the north,’ and ‘the west and the north belong to Yin because of insufficiency of sunshine’. Epidemiological survey indicates that residents in Xinjiang have dryness syndrome to varying degrees. Moreover, dryness is a common cause for multiple diseases with high morbidity, such as allergic rhinitis, dermatosis, bronchitis, and some cardiovascular diseases. Chronic obstructive pulmonary disease (COPD) manifests as a special syndrome type in Xinjiang, or cold-dryness syndrome. Recently, Traditional Chinese Medicine (TCM) epidemiological survey found that the frequency of cold-dryness syndrome in the northwest is 7.7% and the constituent ratio is 9.5%. Previous study found that the body weight of COPD model animals is lower than that of normal animals, which can be exacerbated by cold-dryness. Pulmonary inflammation of model animals manifests as interleukin-1β (IL-1β), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) levels increased, which are higher than that in COPD models. There is no significant changes in interleukin-10 (IL-10) levels. For the general inflammatory response induced by the northwest cold-dryness syndrome, IL-1β is mainly increased. Therefore, cold-dryness has a tendency to exacerbate inflammation of the lungs and body. Moreover, as a stress source, cold-dryness may influence the production and development of many diseases. Homeostasis is maintained continuously by physiologic responses or adaptations in organisms, and excesses or changes in stress may induce disease states. Immune logic function plays an important role in homeostasis, so this experiment was designed to provide a reference for the prevention and treatment of cold-dryness type COPD.

MATERIALS AND METHODS

Experimental animals

Ninety Wistar rats, male, weighing (150 ± 20) g, were supplied by the Center of Experimental Animals, Xinjiang University of Medicine, License No: SCXK (Xin) 2003-0001.

Instruments

Instruments used in the experiment were: FLI-2000H artificial climatic test chamber (EYELA Co., Tokyo, Japan); BUXCO MA1320 respiratory function test table (Buxco, Wilmington, CA, USA); BS-1105 electronic balance (Sai Duo Ke Shi Scales Co. Ltd., Beijing, China); slicer (Leica, Wetzlar, Germany); microscope (Leica, Wetzlar, Germany); 60 cm × 70 cm × 100 cm Yakeli poisoning cabinet (self-made); high-speed centrifuge (Thermo Scientific Forma, Waltham, MA, USA); adjustable micro-pipetter (Eppendorf, Hamburg, Germany); low temperature freezer (Thermo Scientific Forma, Waltham, MA, USA); flow cytometer (Beckman Coulter, Brea, CA, USA); electronic balance (Sartorius, Göttingen, Germany); and micro-pipette (Gilson, Middleton, France).

Drugs and reagents

Drugs and reagents used included: cigarettes (tar content 12 mg, nicotine in smoke 1.0 mg, carbon monoxide in smoke 13 mg, Xuelian brand, Xinjiang Cigarette Factory, Urumqi, China); elastase, 10% formalin solution, and 0.4% pentobarbital sodium (Shanghai Huayi Biologic Science and Technique Co. Ltd., Shanhai, China); CD3 flow cell antibody, CD4+ flow cell antibody and CD8+ flow cell antibody (BD Pharmingen, CA, USA).

Animals and grouping

After the rats were raised adaptively for 2 days and weighed, they were numbered according to body weight order and randomly divided into three groups according to a random number table. The three groups were: COPD model group (n=35), cold-dryness COPD group (n=35), and normal control group (n=20).

Methods of modeling

The rats in the COPD and cold-dryness COPD groups were given an endotracheal dripping of elastase (20 U elastase in 0.8 mL saline for each 100 g body weight) on the 30th day, and were placed in a self-made poisoning cabinet with a volume of 420 L from the 1-29th and 31-90th days. The length, width, and height of the cabinet were 60, 70, and 100 cm, respectively, with an exhaust of 2.0 cm in diameter at the top and a central processing unit fan suspended in the middle, evenly distributing the smoke. There was also a 250 g gel drier placed in the cabinet before the beginning of each smoking. The poisoning cabinet was connected to a smoking apparatus, and the smoke was...
sucked through a three-limb tube and 80 mL feeding equipment into the poisoning cabinet. The smoke was supplied at 15 sucks/min at all times to keep relatively stable concentration. Eighteen rats smoked for 1 h each time. The poisoning cabinet was cleared on the time-interval between the two groups in the smoking with the inlet and the exhaust open, and the residual smoke was dispersed for 5 min by the fan. For the cold-dryness COPD model, rats were placed each night in an artificial climate test chamber at 6°C±1°C and relative humidity 25.0%-32.8%, 10 h each day. All other conditions were the same as the COPD model group. The rats in the control group were given an endotracheal dripping of the same volume of saline with the same method as the model group on the 30th day.

Before operation, instruments were disinfected with high pressure steam (121°C, 120 kPa, 30 min). After anesthetization with 0.4% sodium pentobarbital, 50 mg/kg body weight, rats were fixed on a plate and a BD Intima-Ⅱ puncture trocar (0.7 mm×19 mm, Becton Dickinson Medical Devices Co., Ltd., Suzhou, China) was slowly inserted along the tongue into the trachea and then the needle was withdrawn. A 1 mL needle tube containing the needed dose of elastase was used with the head at a high position and the tail at lower position. The needle was angled towards the left at 45°, and a half of the volume of the elastase solution was slowly injected while the other half of the elastase solution was injected with the needle towards the right. Then 1 mL of air was injected pushing the elastase solution completely into the lung. Then, the back of the rat was patted spreading the elastase solution evenly throughout the lung. Rats were kept warm and after waking were placed in a cage.

**Determination of pulmonary function of the rat**
Peak expiratory flow (PEF), time of inspiration (Ti), time of expiration (Te), and 50% tidal volume expiratory flow (EF50) were detected by a non-invasive small animal pulmonary function analysis system.

**Preparation of pathologic specimens of lung tissues**
Rats were anesthetized with abdominal injection of 0.4% sodium pentobarbital, 50 mg/kg body weight, and killed by exsanguination from the inferior vena cava. Lungs were rapidly removed and the mid lobe of the right lung and part of the trachea and bronchus were placed in 10% formal for fixation, routinely dehydrated, immersed in wax, embedded, and sectioned (3 continuous slices of 4 μm for each lung tissue). Sections were stained with HE, and the morphological changes of the tissue were photographed and observed under a microscope.

**Detection of peripheral blood CD4+, CD8+, and CD4+CD8+**
On the ninetieth day after ending the experiment, 6-7 mL blood was taken from femoral artery and poured into a tube with EDTA. Then, 100 μL of anti-coagulated whole blood was poured into a flow-type tube with 20 μL of antibodies against CD3, CD4+, and CD8+. The tube was incubated for 30 min at room temperature, in darkness. Red cells were lysed with hemolysin, centrifuged at 1000 ×g for 5 min, and collected. Cells were then washed twice with 2 mL PBS, centrifuged at 1000 ×g for 5 min, and then collected and resuspended in 300 μL PBS. Cells were finally detected with the flow cytometer.

**Data analysis**
Continuous variables are expressed as (mean±standard deviation). All samples were tested to ascertain if they followed a normal distribution. The sample data have a normal distribution. Data comparison among groups was performed using ANOVA and and Homogeneity of Variance Tests. Comparison between groups was carried out using the independent samples t-test. SPSS Version 11.5 (SPSS Inc., Chicago, IL, USA) was used for data analyses. *P*<0.05 was considered significant.

**RESULTS**

**Gross observation of lung**
On the 90th day after ending the experiment, in the normal group, both lungs showed a pink, smooth surface, and rapidly shrank after opening the thoracic cavity. In the COPD model group, the lung was obviously expanded with pallor, there was an increased volume compared with the normal group, and the white foam area could be seen on the surface of the lung. The elasticity was also weakened. In the cold-dryness COPD group, the lung was also expanded with pallor, and the volume was significantly increased as compared with the normal group. The white foam area could be seen on the surface of the lung with some black spots, and elasticity was decreased (Figure 1).

**Comparison of pathologic morphologies among the groups**
On the 90th day after ending the experiment, in the normal group, the size of the alveoli was uniform and the thickness of alveolar wall was normal. In the periphery, inflammatory cell infiltration was not seen or was sparse (Figure 1A). In the COPD group, there was alveolar expansion and narrowing of the septum. The septum was also broken and fused into larger bursal lumens, with congestion and inflammatory cell infiltration (Figure 1B). In the cold-dryness COPD group, alveoli expanded and the septum was narrowed. The septum was also broken and fused into larger bursal lumens, with congestion and inflammatory cell infiltration (Figure 1C).

**Comparison of pulmonary functions among the groups**
On the 90th day after ending the experiment, the PEF in the cold-dryness COPD group was lower than that
in the COPD group and the normal group (P<0.01). Ti in the cold-dryness COPD group was higher than that in the COPD and normal groups (P<0.05). Te in the cold-dryness COPD group was higher than that in the COPD and the normal groups (P<0.01). Moreover, the COPD group was higher than that in the normal group (P<0.01). EF50 in the cold-dryness COPD group was lower than that in the COPD and the normal groups (P<0.01), and the COPD group was lower than that in the normal group (P<0.05) (Table 1).

**Comparison of immunologic functions among the groups**

On the 90th day after ending the experiment, In the peripheral blood, the CD4+ content the cold-dryness COPD group was lower than that in the normal and COPD groups (P<0.01). CD8+ content the cold-dryness COPD and COPD groups were higher than that in the normal group (P<0.01). Moreover, CD8+ content in the cold-dryness COPD group was higher than that in the COPD group (P<0.01). CD4+/CD8+ in the cold-dryness COPD and COPD groups were lower than that in the normal group (P<0.01), and CD4+/CD8+ in the cold-dryness COPD group was lower than that in the COPD group (P<0.05) (Table 2, Figure 2).

**DISCUSSION**

The lungs of rats in the COPD model group and cold-dryness COPD group showed obvious expansion with pallor. Moreover, the volume of the lung was significantly increased as compared with the normal group, and the white foam region on the lung surface could be seen along with decreased elasticity. In the cold-dryness COPD group, some black spots were observed on the lung. Pathologic observation of the lung showed that in both the COPD group and the cold-dryness COPD group, alveoli expanded and septa narrowed, broke, and fused into larger bursal lumens. There was also congestion, inflammatory cell infiltration, proliferation, and thickness of smooth muscle below the small bronchus, all of which conform to clinical pathologic changes of COPD. At the same time, in the model groups, PEF decreased, Ti and Te increased, and EF50 decreased, which also conform to the clinical characteristics of COPD.

![Figure 1 Comparison of pathologic morphologies among groups (hematoxylin-eosin staining, ×100)](image-url)
T-lymphocytes are not only the effector cells of cellular immunity, but also important immunoregulatory cells. Among them, helper T lymphocyte CD4+ cells and inhibitory T lymphocyte CD8+ cells have important regulatory actions on cellular immunity and humoral immunity. Because the regulatory action of T cells is completed mainly by CD4+ and CD8+, normal immune function is dependent on maintaining a certain ratio of the T cell subgroups. In particular, the relative stability of the CD4+/CD8+ ratio is important. Coordination of both maintains normal immune response. Increase in total T cell count (CD3) including CD4+ and CD8+ occurs on the alveolar wall during pulmonary emphysema, but CD8+ cells play a leading role. Increased CD8+ cell count also occurs in the large and small airway walls and smooth muscle of the peripheral air passages in COPD patients. A main function of CD8+ is to fight cytolysis induced by viral infection and apoptosis. A decrease in the CD4+/CD8+ ratio is one of the markers of severity and unfavorable prognosis of the disease. CD4+ cells can express correlative antibodies of inhibitory or active natural killer cells, recognize corresponding ligands, and conduct inhibitory or active signs. These effects influence the functions of CD4+ helper T cells to induce and maintain an anti-viral effect of CD8+.

More study is being conducted on the relation of COPD to immunological function. Immunological responses of the organism are commonly carried out by various lymphocytes and cytokines. It is found from immunohistochemical observation that in the patients with COPD, CD4+ and CD8+ have significant changes in the trachea before air passage inflammation. In this study, it was found that cold-dryness could further exacerbate the decrease of CD4+ cells and the increase of CD8+ cells and lead to reversion of CD4+/CD8+. 

Figure 2 Results of CD4+ and CD8+ among groups
A-C: normal group; D-F: COPD group; G-I: cold-dryness COPD group. Rats in the COPD and cold-dryness COPD groups were given an endotracheal dripping of elastase, while in the normal control group were given an endotracheal dripping of saline on the 30th day. COPD: chronic obstructive pulmonary disease.
Cold-dryness can also decrease PEF, and increase Ti and Te. In air passage inflammation of patients with COPD, T-lymphocytes have immune function and regulate immunity.\textsuperscript{26} Reversion of CD4+CD8+ disorder of immune function of T-lymphocytes, and immune injury are important factors of COPD disease advancement. Moreover, the increase of CD8+ in the peripheral air passages in COPD patients is related to limitation of air flow.\textsuperscript{26} This experiment found that in COPD rats, Te and Ti increased, with Te being the main increase.

In brief, cold-dryness can exacerbate CD8+ cell increase and decrease the CD4+/CD8+ ratio in COPD model rats. The effects of cold-dryness on lung function are mainly reflected in prolongation of time of expiration and decrease in 50% tidal volume expiratory flow. These factors are possibly a cause of the north-west cold and dry environment leading to chronic obstructive lung disease.

REFERENCES